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13. ABSTRACT (Maximum 200 words)

The support from the US Army Research Office has allowed us to develop novel instrumentation and methods to use state-of-the-art atomic force microscopy in biological applications. We have developed several methods to facilitate high-resolution structural studies of supported membranes, revealing their structural and functional behaviors that have eluded being discovered with other conventional methods. These studies have also established a solid foundation for our structural elucidation of molecular level conformation of membranous bacterial toxins, such as cholera toxin and α-hemolysin. In collaboration with Prof. Hagan Bayley, we have shown that two mutants of α-hemolysin assembled into heptamer in biologically relevant conditions. The combination of molecular engineering and in situ high-resolution structural elucidation shows a great promise in future success of tackling difficult biological problems. Our progress in the study of supported membranes has allowed us to investigate the behavior of genetic materials when they interact with bilayer membranes. We have found the two-dimensional condensation of DNA on cationic lipid bilayers and a strong binding of DNA to zwitterionic lipid bilayers. These results provide structural information in our understanding of the mechanisms that govern the packing of genetic materials in cellular organisms and the process of gene delivery trials.

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Technical Reports

Introduction

It has been realized that elucidation of the molecular structure of membrane proteins remains difficult with conventional imaging, spectroscopic, and scattering techniques, such as electron microscopy, NMR imaging technique, and crystallization for X-ray or electron diffraction. The advent of surface topography probing atomic force microscope (AFM), in particular with the capability of operating in buffer solutions, opens new avenues in molecular resolution structural studies of membrane proteins in situ. With the support from the US Army Research Office, we have developed novel instrumentation and methods that have enabled us to study the molecular structural characteristics of several membrane proteins, bacterial toxins α-hemolysins and cholera toxin. Our methodology developments have also revealed several structural and functional characteristics of supported membranes, establishing a solid foundation for our structural studies of the bacterial toxins. In related studies, we have found that DNA can assemble on bilayer membranes, with condensed stuctures on cationic lipid bilayers, and less ordered stuctures on zwitterionic lipid bilayers. These results contribute greatly to the advancement in basic scientific research in the frontier of high-resolution structural studies of membrane proteins and in studies of membrane-membrane and membrane-protein interactions. In the following, we will describe briefly details of our achievements.

Supported membranes: structure and function

The lipid bilayer is basic structural framework of biological membranes. It houses membrane proteins such as channels and ion-pumping ATPases and membrane receptors which regulate various cellular and sub-cellular functions and activities. In order to understand the properties of biological membranes, it is therefore of great importance to study the basic structural framework, the lipid bilayer. For this purpose, model phospholipid membranes have been widely used. For structural studies of membranes and membrane proteins, supported bilayers are convenient hosts. Conventional characterizations of planar supported membranes employ optical and spectroscopic methods, with the former approaches revealing structural features at sub-micron resolution and the latter means providing averaging results of large ensembles. Recent developments in atomic force microscopy (AFM) have shown the possibility of *in situ* high-resolution structural studies of supported membranes. In the following we show several characteristics of supported bilayers found in our studies.

a. Bilayer-bilayer interaction induced ripple formation

The ripple phase in phosphatidylcholine (PC) bilayers has a distinct structural characteristic, in which the lipid bilayer has a regular spatial modulation. Electron microscopy and X-ray diffraction on multilamellar PC bilayers show that typical wavelengths of bilayer ripples are 12 to 16 nm or double that value.

Several theoretical models have been proposed to understand the mechanism of the ripple-formation. Several theoretical models suggest that the ripple structure may exist in an unilamellar bilayer as a result of some lipid-lipid interactions in the bilayer. However, bilayer-bilayer interactions have been also conjectured to play a role in the ripple-formation. Because of the complication of a lipid bilayer, these theoretical modeling studies may not account for all factors in experiments on different bilayers under various conditions.

We have developed a method to prepare supported double-bilayers on mica by the fusion of vesicles. This method facilitates the comparison of structural features on top of the double-bilayer with those on top of the unilamellar bilayer, and the results of which may provide information about the bilayer-bilayer interaction. The use of low salt solution (20 mM NaCl) minimizes any effect due to ions on the ripple-formation in our experiments. The capability of operating AFM in solution allows us to study the structure of supported bilayers with *in situ* AFM. For supported dipalmitoylphosphatidylcholine (DPPC) bilayers, we detected the existence of a ripple structure in double-bilayer regions only, indicating that the bilayer-bilayer interaction may play a role in the formation of the ripple structure in our systems. A majority of the ripple structure has an average wavelength of 27 nm and average amplitude of 0.5 nm (we call it the "normal" ripple structure). Some large ripples, often coexisted with the "normal" ripples, have an average wavelength of 53 nm and largely fluctuated amplitudes of an average of 8 nm. The amplitude and the fluctuation of the large ripples decrease as the probe force increases, and the amplitude of the "normal" ripples does not change for probe forces as large as 3 nN. We also observed an extremely slow relaxation of the ripple structure. The ripple structure can be induced repeatedly, but the pattern of formed ripples varies under different initial conditions. Although double bilayers with a high surface coverage are reliably formed, our method does not result in the formation of supported triple or multilamellar bilayers. Fig. 1 shows an example of a unilamellar DPPC bilayer and an example of ripples on supported double bilayers.

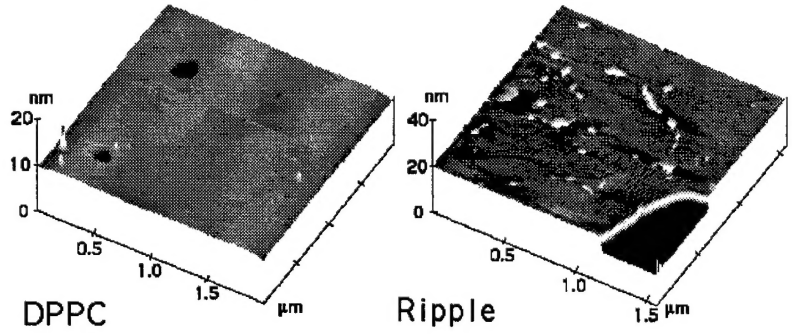

Fig. 1. A planar unilamellar DPPC bilayer with several bilayer defects and a ripple structure on a double bilayer.

b. Lipid loss process in supported bilayers

One prominent characteristic of lipid bilayers is the dynamic nature of lipids in the bilayers. Thus far, extensive studies on the dynamic properties of phospholipids have revealed much information about the organization and the function of these lipids in biological membranes. Thermodynamically, free monomeric lipid molecules coexist with lipid bilayers in aqueous solution [9,10], although the population of the former is much less, as a result of the hydrophobic interaction between lipid molecules which drives the assembly of lipid bilayers. Light scattering measurements and uses of fluorescent, radioactive, and spin labels have facilitated the study of the lipid-exchange process and have shown that it has a rather long thermal equilibrium time, with a time constant of the order hours around 37 8C. The lipid-exchange process involves the transfer of monomeric lipid molecules from the bilayer to the solution and *vice versa*. The rate of the lipid exchange has an Arrhenius behavior, with a barrier in the range of 30 - 50 kT, where kT is the thermal energy at room temperature.

AFM allows possible correlation of functional studies to local variations in supported membranes. Specifically, the dynamic process of the lipid-exchange process can be studied by measuring the loss of lipids and monitoring accompanying structural changes in bilayers. We found that the loss of lipids is characterized by the increase of defect areas. Measurements of defect areas from a large number of AFM images provide better statistics in calculating the lost amount of lipids in the bilayers. We have also found some interesting structural features associated with the lipid-loss process. Interdigitated membrane domains are induced in the lipid-loss process at temperatures above 45 °C and disappear at later times as the area of bilayer defects increases. Most of these domains are in contact with some bilayer defects. The rate of the lipid loss is extremely slow and has an Arrhenius behavior with an activation energy of 37 kT.

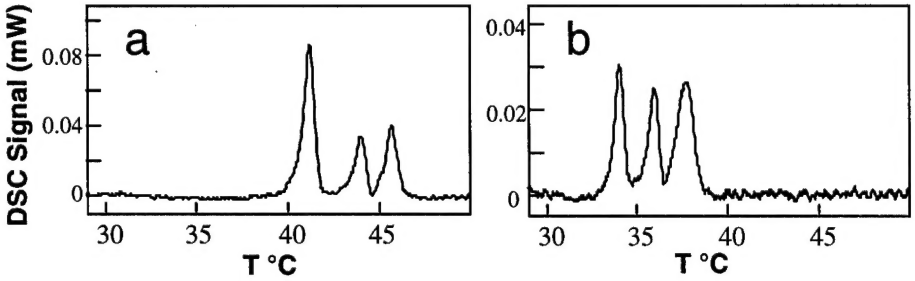

Fig. 2. The DSC scans showing the main phase transitions of supported bilayers. Part a show the phase behavior of supported DPPC bilayers, with the transition around 41 °C representing the main phase transition of lemellar bilayers and the double transition at higher temperatures resulting from the two leaflets of supported bilayers. Part b shows the similar phase behavior of supported DiC15-PC bilayers, with corresponding main phase transitions at lower temperatures.

a. The main phase transition of supported bilayers

One prominent feature for a 2-D phospholipid membrane is that there are several equilibrium phases in different temperature ranges. It is expected that these phases should also exist in supported membranes. A convenient method to detect the phase transitions employs a differential scanning calorimeter (DSC). However, for supported membranes, statistically insignificant accessible surface area poses a serious challenge to detect phase transitions with a DSC. To overcome this shortcoming, we chopped a piece of mica into microscopic chips and used them as substrates. This method significantly increases accessible surface area and facilitates studies of the main phase transition of supported bilayers with a DSC. We have found that the main phase transition temperature for supported bilayers increases by several degrees compare to that of lamellar bilayers. Moreover, our data show that the two leaflets of supported bilayers are decoupled, with two main phase transition temperatures separated by about 2°C. Fig 2 shows our results of DSC scans of supported bilayers of two kinds of PC bilayers and they show clearly the unexpected features of the phase behavior of supported bilayers.

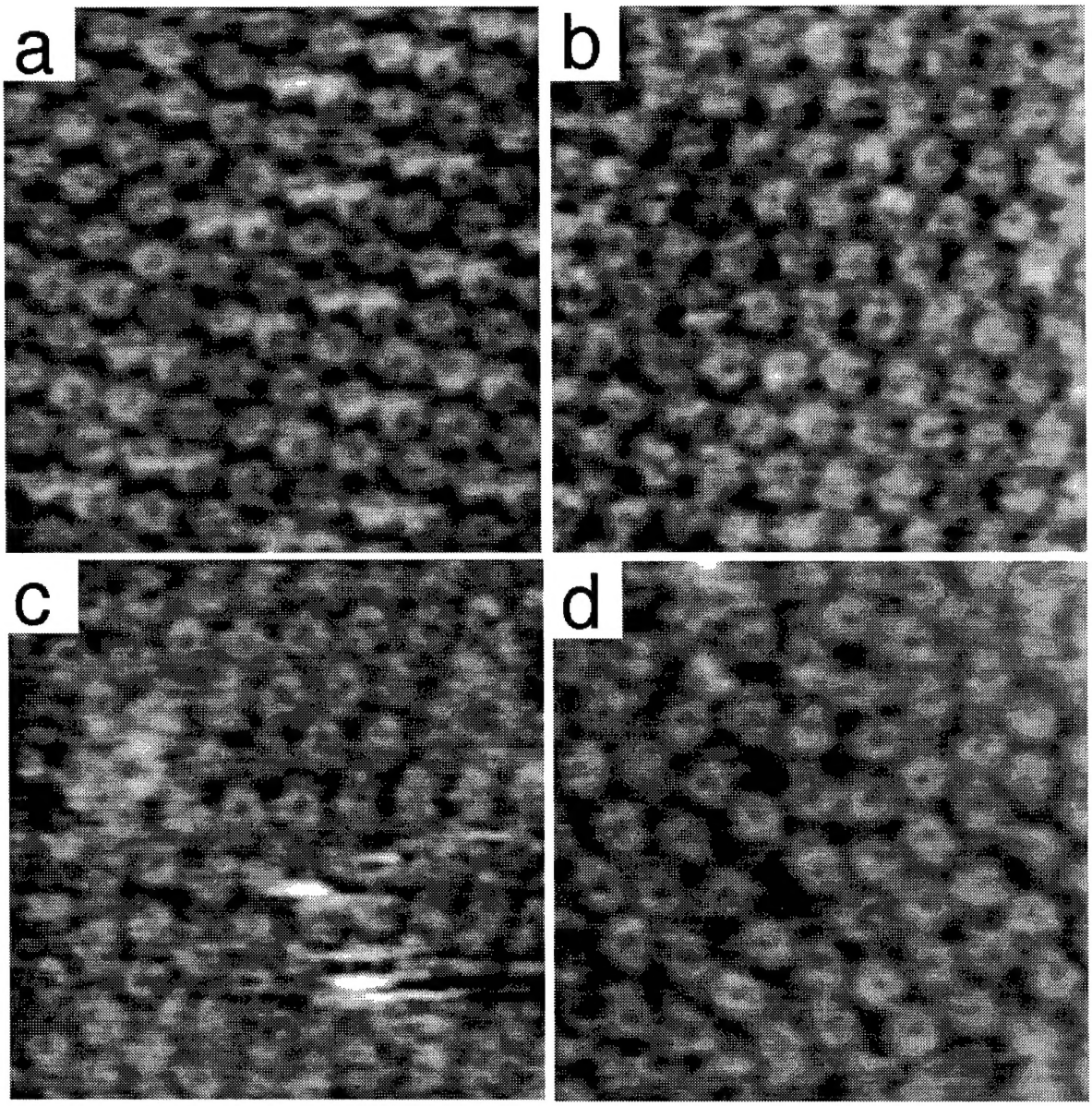

Fig. 3 Four images of ctB on four bilayers containing the ctB receptor G_{M1}. These bilayers are: (a) egg-PC; (b) DLPE; (c) Dic15-PC; and (d) DPPC.

Molecular structure of membrane proteins

The key to yield nm resolution on biological specimens with *in situ* AFM imaging is to use flat solid support and to obtain high density surface coverage of biomacromolecules on the support. Thus, supported bilayers are essential hosts of membrane proteins to allow high-resolution *in situ* AFM imaging since these bilayers have properties very close to those of cellular membranes and most cell membranes are 2-D fluid host for membrane proteins. In the following we summarize our structural studies of cholera toxin B-oilgomers and α-hemolysin (αHL) prepores on supported bilayers, as well as a mutant of α-hemolysin on mica in solution. For high-resolution molecular structure, we also employed rotational average to enhance signal-to-noise ratio.

a. The pentameric structure of cholera toxin B-oligomer on receptor incorporated bilayers

For high-resolution imaging of cholera toxin B-oligomer (ctB) on receptor-incorporated supported bilayers, we first prepared supported bilayers made of different lipids by the vesicle-fusion method. Then, 50 μM gangliosides GM1, the the membrane receptor for ctB, in 20 mM NaCl are incubated with the supported bilayers for about 30 min at temperatures about 10 degree above the main phase transition temperature of the corresponding bilayers. This procedure incorporated the receptor into supported bilayer. Afterwards, the bilayer was incubated with 0.2 μM ctBs for a period from about 40 min to several hours at room temperature. Excess ctBs were removed by washing.

Figure 3 shows 4 typical images of membrane-bound ctBs on four different bilayers. Here, the G_{M1} concentration on the upper bilayer-leaflet should be at least about 10 mol%, judging from the full surface coverage of membrane-bound ctBs in all images. These images are stable under repeated high-resolution scans. We also see that the molecular structure of ctBs shows some degree of randomness in their orientation on the membrane. Some proteins only show a ring-like structure without resolving the pentamer.

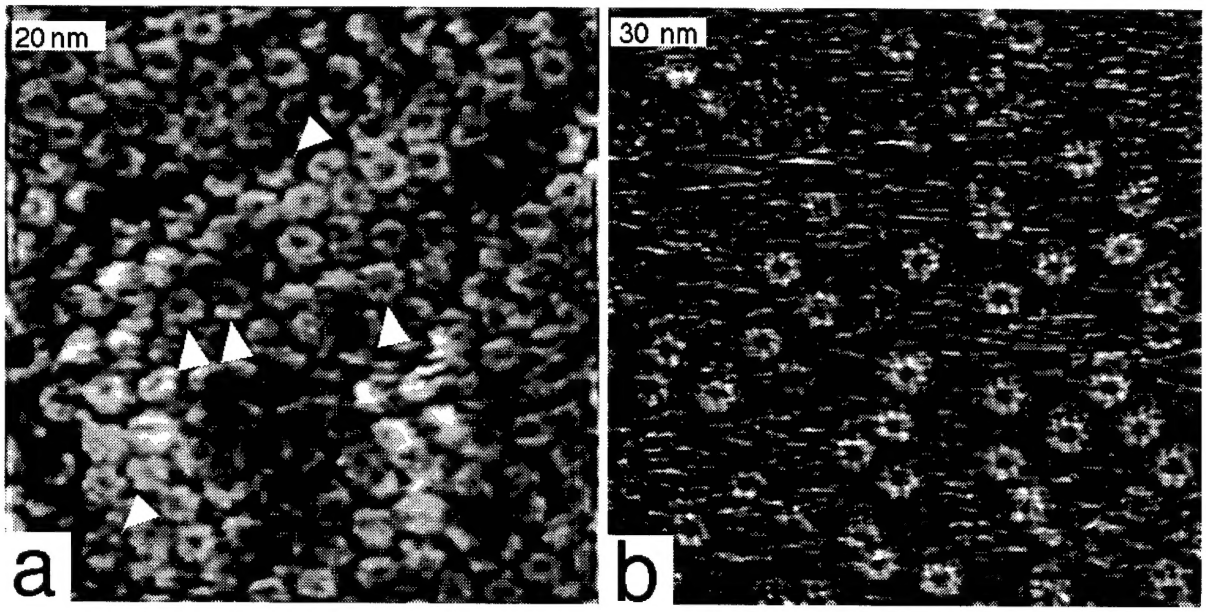

Fig. 4 shows typical images of two αHL mutants in buffer solutions with (a) αHL-H5 locked in the prepore on an egg-PC bilayer and (b) assembled TCM on mica. Arrows in (a) indicate directly resolved heptamers.

b. The heptameric structure of α-hemolysin prepore

In collaboration with Prof. Hagan Bayley, we have imaged self-assembled structures of two mutants of α-hemolysin (αHL) with in situ atomic force microscopy. Using supported fluid phase egg-PC bilayers, we were able to show, for the first time, that an αHL mutant, αHL-H5, assembled into heptamers upon binding to membranes when the condition locked the assembly process at the prepore state. This result shows the prepore is indeed a heptamer, in support of the step-wise assembly model. The incubation time (5 - 7 days) to achieve high-density surface coverage of prepores on egg-PC, however, was much longer than that on cellular and vesicular

membranes. Fig. 4a shows an example of αHL-H5 prepores on egg-PC, with many individual heptamers directly visible.

c. The heptameric structure of a truncated α-hemolysin mutant (TCM)

We have also studied the structure of a different mutant of αHL, TCM, with the protein also provided by Prof. Baylay. This mutant was obtained by replacing 25 amino acids of the central domain with the sequence Asp-Gly. This particular mutant already self-assembles into molecular complexes in solution because of the removal of the 25-residual transmembrane domain. The addition of Asp-Gly promotes the formation of a type I' β-turn. These molecular complexes were adsorbed to a mica surface in solution and imaged with AFM *in situ*. Fig. 4b shows an example. It is clear that the molecular complex is a heptamer. This shows that the heptamer is the preferred functional form under natural conditions.

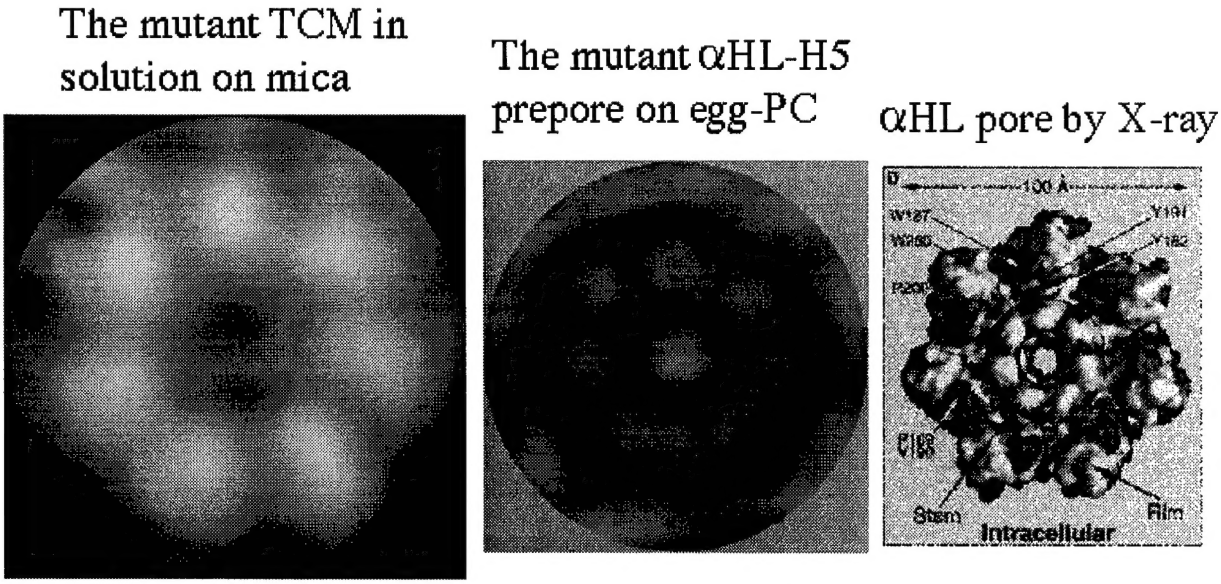

Fig. 5. Averged images of two α-hemolysin mutants from AFM studies and a top view image of the wild type α-hemolysin from X-ray diffraction. All images are at the same scale.

d. Rotational imaging average and the molecular structure of α-hemolysin mutants

To enhance signal-to-noise ration, we performed rotational imaging averaging on α-hemolysin mutants. Briefly, individual oligomers were zoomed out from original AFM images so that the center of the oligomer coincided with the center of the zoomed image. Each oligomer was aligned with respect to a reference chosen from one of the oligomers, by finding the minimum in the *chi-square* distance between the two images as the first image was rotated in 360 one degree steps. The *chi-square* distance is defined as:

$$D^2(d1, d2(\theta)) = D\sum_{i,j}(D_{d1}(i,j) - D_{d2}(i,j))^2 \qquad (1)$$

in which D is a normalization constant, $D_{d1}(i, j)$ represents the data at (i, j) of the reference image, and $D_{d2}(i, j)$ represents the data at (i, j) of the oligomer image at a rotation angle θ. Then, the averaged image was used as the reference to realign individual oligomers again, and so forth. We found that the alignment converged after the second average. Because of the unknown

extend of compression as the tip scans the sample, the height of the oligomer obtained from AFM images may not reflect the true dimensions of the molecule. Thus, we have not attempted to obtain molecular dimensions in the vertical direction.

The averaged results for both mutants are shown in Fig. 5, along with a top-view structure of the wild-type α-hemolysin from X-ray diffraction. The structure for both mutants indicates that the 7-fold axis is perpendicular to the imaging surface. Thus, for the prepore, the orientation of the protein complex has the symmetry axis perpendicular to the membrane plane. The average image is of the same scale as the X-ray result, indicating close structural similarity of the prepore and the pore in their molecular organization. The dimension of the mutant TCM, though, is larger than that from X-ray results of wild type aHL.

The assembly of genetic materials on bilayer membranes

The packing of DNA in cellular organisms involve dimensionality reduction and condensation. Moreover, the condensation of DNA has been shown to promote increasing activities in various biological processes, including gene-recombination and strand-exchange, DNA polymerization and ligation, catenation of circular plasmid DNA, and replication. It is therefore of great importance to study in *vitro* DNA condensation for the understanding of the molecular level details of the above biological processes.

Studies of how DNA interacts with lipid bilayers is important for understanding how foreign genetic materials can be efficiently delivered into cells. Cationic lipids have been favorable materials to mediate gene delivery, in which the first stage is the formation of DNA-cationic lipid complexes that are granules of average diameter about one micron.

We have studied structural characterstics of how DNA interacts with lipid bilayers. DNA molecules adsorbed on cationic lipid bilayers are higly condensed. Thus, we have a model system to study the basic science of the condensation of DNA. We have also found that DNA molecules can also bind strongly to zwitterionic lipid bilayers, providing clues toward an understanding of the gene delivery process that may be useful for gene therapy researches.

a. 2-D condensation of DNA on cationic lipid bilayers

The cationic lipids we have used involve DPDAP that has a broad main phase transition (T_m ~ 29 8C) and DSDAP that has a sharp main phase transition (T_m ~ 38 8C). On DPDAP, the condensation of DNA onto the 2-D membranes is independent of the length of DNA and disregard whether the DNA molecules are circular or linear. On DSDAP, the condensation is facilitated when incubating DNA with the bilayer was at a temperature above the main phase transition temperature of DSDAP. Fig. 6 shows two examples of condensed DNA on DPDAP. We note that the resolution is high enough to resolve the pitch of DNA on a number of DNA segments directly.

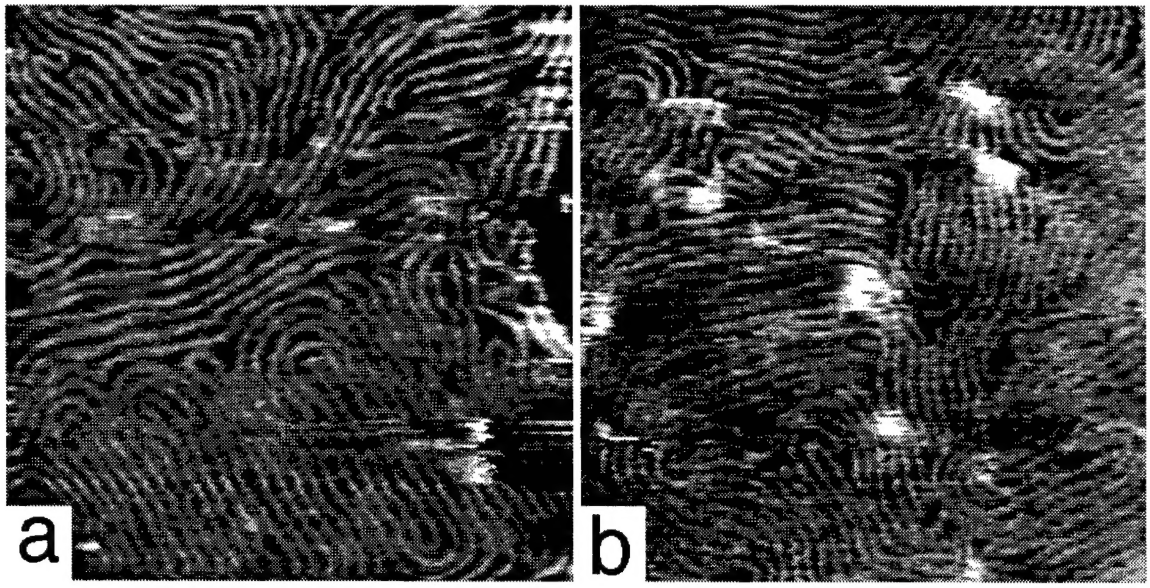

Fig. 6. Condensed DNA on cationic lipid bilayers with (a) a plasmid DNA and (b) linear DNA fragments.

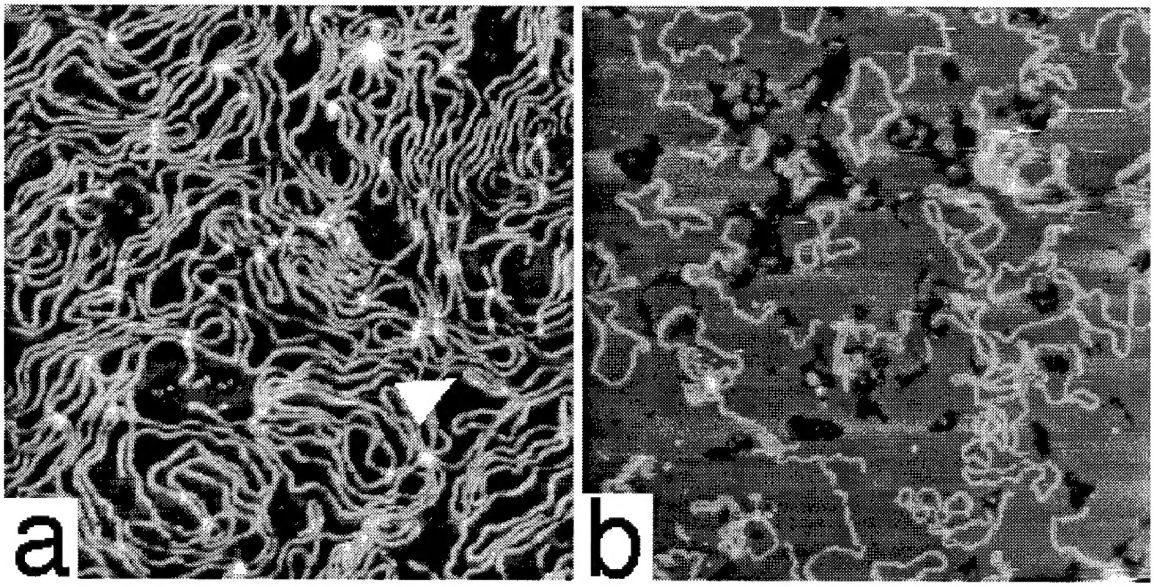

Fig. 7 Two typical images of DNA on DPPC with (a) of an area 1 μm × 1 μm and (b) of an area of 2 μm × 2 μm.

a. The assembly of DNA on zwitterionic lipid bilayers

The zwitterionic lipid for DNA adsorption is DPPC. Fig. 7 shows two examples of DNA molecules on DPPC bilayers. The ability of stable high-resolution imaging DNA on DPPC in solution indicates that the binding of DNA to DPPC bilayers must be fairly strong. That the surface coverage of membrane-bound DNA can be reduced by brief incubation with 1 M NaCl

solution indicates that the electrostatic interaction between DNA and the large dipole moment of DPPC headgroup plays a major role to tether DNA.

Our results may provide structural information at the molecular level toward an understanding of the pathways in gene delivery trials. We show that DNA binds to zwitterionic lipids, although not so strongly as it binds to cationic lipids. Thus, cellular membranes that contain large amount of zwitterionic lipids would not repel, but rather, attract foreign genetic materials and facilitate their intracellular delivery. Experimental investigation has found that using mixtures of PC and cationic lipids to complex with DNA resulted in higher delivery efficacy, suggesting the involvement of membrane fusion in the gene delivery process because zwitterionic lipids are more suitable to fuse with cellular membranes. The requirement of cationic lipids indicates the need of stronger binding of DNA and, most likely, the involvement of DNA condensation in the complexes to catalyze the gene delivery.

Summary

In summary, we have demonstrated that the power of the novel AFM instrumentation is the key to recent developments in high-resolution in situ structural studies of membrane structures and membrane proteins. Exciting new results are to be expected with further developments in instrumentation and methodology.

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Jie Yang, Symposium, ACS Meeting, August 26-27, 1998, Boston.

List of all participating personnel

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Ye Fang, Ph.D. Postdoc Research Associate.
M.S. Malghani, Ph.D. Postdoc Research Associate.
Sean Hand, Graduate Student.